

RESEARCH PAPER

Imipramine as an alternative to formamide to detubulate rat ventricular cardiomyocytes

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Abstract

Cardiac T-tubules are membrane invaginations essential for excitation-contraction coupling (ECC). Imipramine, like other cationic amphiphilic drugs, interferes with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) interactions with proteins maintaining the tubular system connected to the cell surface. Our main purpose was to validate imipramine as a new detubulating agent in cardiomyocytes. Staining adult rat ventricular myocytes (ARVMs) with di-4-ANEPPS, we showed that unlike formamide, imipramine induces a complete detubulation with no impact on cell viability. Using the patch-clamp technique, we observed a ~40% decrease in cell capacitance after imipramine pretreatment and a reduction of $I_{Ca,L}$ amplitude by ~72%. These parameters were not affected in atrial cells, excluding direct side effects of imipramine. β -Adrenergic receptor (β -AR) stimulation of the remaining $I_{Ca,L}$ with isoproterenol (Iso) was still effective. ECC was investigated in ARVMs loaded with Fura-2 and paced at 1 Hz, allowing simultaneous measurement of the Ca²⁺ transient (CaT) and sarcomere shortening (SS). Amplitude of both CaT and SS was decreased by imipramine and partially restored by Iso. Furthermore, detubulated cells exhibited Ca²⁺ homeostasis perturbations. Real-time cAMP variations induced by Iso using a Förster resonance energy transfer biosensor revealed ~27% decreased cAMP elevation upon β -AR stimulation. To conclude, we validated a new cardiomyocyte detubulation method using imipramine, which is more efficient and less toxic than formamide. This antidepressant agent induces the hallmark effects of detubulation on ECC and its β -AR stimulation. Besides, we provide new insights on how an imipramine overdose may affect cardiac function and suggest that PI(4,5)P₂ is crucial for maintaining T-tubule structure.

1 | INTRODUCTION

Transverse tubules (T-tubules) of cardiac myocytes are invaginations of the surface membrane that form a complex interconnected network penetrating deep into the cell interior (Hong & Shaw, 2017). These tubular structures are found mostly in ventricular myocytes where they occur near the sarcomeric z-discs (Caldwell et al., 2014), allowing functional junctions with the sarcoplasmic reticulum called dyads. They are critical for excitation-contraction coupling (ECC) by concentrating L-type calcium channels (LTCCs) and positioning

them in close proximity to ryanodine receptor (RyR) clusters at the junction of the sarcoplasmic reticulum. During an action potential, T-tubules propagate the cell-membrane depolarization inside the cell, allowing Ca²⁺ entry to trigger successive Ca²⁺ releases, thus promoting synchronicity as well as efficiency of the Ca²⁺-induced Ca²⁺ release (CICR) phenomenon (Fu et al., 2016). Their enrichment with ion channels and the presence of signalling pathway components such as β -adrenergic receptors (β -ARs) (Nikolaev et al., 2010) and adenylyl cyclases (AC) (Timofeyev et al., 2013) make these structures essential for cardiomyocyte function and regulation. Alterations

in their morphology and density are believed to participate in the development of heart failure (Hong & Shaw, 2017), and thus understanding their role is crucial and may open new therapeutic avenues.

To define the role of the T-tubules in the physiology of ventricular cells, Orchard's group in the late 1990s provided a simple detubulation method. It is based on an osmotic shock induced by a high concentration of formamide followed by its quick removal to disrupt the normal coupling of the membrane invaginations with the cell surface (Brette, Komukai, & Orchard, 2002; Kawai, Hussain, & Orchard, 1999). Since then, dozens of reports have been published using this technique as a gold standard method to detubulate cardiomyocytes. However, despite the apparent preservation of the microtubule network (Brette et al., 2002), formamide incubation and removal produce drastic changes in cell volume (Kawai et al., 1999), which may alter subcellular microdomains. Furthermore, although quite efficient, detubulation by formamide is incomplete, with ~20% of the cells remaining intact (Kawai et al., 1999; Pasek et al., 2008). Additionally, ~80% of the myocytes still exhibit a faint staining of invaginations (Brette et al., 2002). More importantly, formamide-induced detubulation is generally variable and more importantly is accompanied by the loss of a large proportion of viable cells, which is especially prejudicial when performed on cardiac cells isolated from valuable pathological or genetically modified models. Therefore, an alternative treatment that is more efficient and less toxic would be useful for cardiac physiologists. Interestingly, a recent study demonstrated that high concentrations of cationic amphiphilic drugs (CADs) can produce detubulation of adult rat ventricular myocytes (ARVMs) probably by interfering with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) interactions with cytoskeletal and Bin-amphiphysin-Rvs (BAR) domain-containing proteins (Osman, Taylor, Allcock, Rainbow, & Mahaut-Smith, 2016). However, a comparison of this detubulation method with the formamide-induced removal of T-tubules has not yet been performed and the consequences for CICR and β -AR signalling of the detubulation induced by CAD binding to PI(4,5)P₂ in ventricular myocytes are unknown. Our aim here was therefore to provide this information. To do so, we selected one of the CADs causing cardiomyocyte detubulation, an antidepressant agent named imipramine (Osman et al., 2016).

2 | METHODS

2.1 | Ethical approval

All experiments were carried out according to the European Community's guiding principles on the care and use of animals (2010/63/UE, 22 September 2010), the local Ethics committee (CEE26 CAPSud) guidelines and the French decree no. 2013-118, 1 February 2013 on the protection of animals used for scientific purposes (JORF no. 0032, 7 February 2013 p2199, text no. 24). Animal experiments were approved by the French Ministry of Agriculture (prefectural agreement no. 2016-108 and agreement to our animal facility no. C 92-019-01). Rats had access to water and standard

New Findings

- **What is the central question of this study?**

Can imipramine, an antidepressant agent that is a cationic amphiphilic drug that interferes with the phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) interactions with proteins maintaining the tubular system, be validated as a new detubulating tool?

- **What is the main finding and its importance?**

Imipramine was validated as a more efficient and less toxic detubulating agent of cardiomyocytes than formamide. New insights are provided on how PI(4,5)P₂ is crucial to maintaining T-tubule attachment to the cell surface and on the cardiotoxic effects of imipramine overdoses.

rat chow *ad libitum* and were housed in a well-ventilated, specific pathogen-free, temperature-controlled environment to ensure that they were not under stress. Enrichment such as sizzle-nest and nest-pack was added in animal cages.

2.2 | Reagents

Isoproterenol from Sigma-Aldrich (Saint-Quentin, France) was prepared in a 1 mg ml⁻¹ ascorbic acid solution at 10 mM (Sigma-Aldrich, Saint-Quentin, France). Di-4-ANEPPS (pyridinium, 4-(2-(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-, hydroxide), formamide and imipramine were from Sigma-Aldrich.

2.3 | Cardiomyocyte isolation and culture

Male Wistar rats (250–300 g) obtained from Janvier Labs (Saint Berthevin, France) were terminally anesthetized by intraperitoneal injection of pentobarbital (0.1 mg g⁻¹) and hearts were excised rapidly. Individual ARVMs were obtained by retrograde perfusion of the heart as previously described (Mika et al., 2013). For enzymatic dissociation, the hearts were perfused at a constant flow of 6 ml min⁻¹ at 37°C for 5 min with a Ca²⁺-free Ringer solution containing (in mM): NaCl 117, KCl 5.7, NaHCO₃ 4.4, KH₂PO₄ 1.5, MgCl₂ 1.7, D-glucose 11.7, Na₂-phosphocreatine 10, taurine 20, Hepes 21, pH 7.1. This was followed by a perfusion at 4 ml min⁻¹ for 40 min with the same solution containing 1 mg ml⁻¹ of collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) plus 300 μ M EGTA and CaCl₂ to adjust free Ca²⁺ concentration to 20 μ M. The ventricles and atria were then separated, finely chopped and gently agitated to dissociate individual cells from each tissue. The resulting cell suspension was filtered on gauze and the cells were allowed to settle down. The supernatant was discarded and cells resuspended four more times with Ringer solution at increasing [Ca²⁺] from 20 to 300 μ M. Freshly isolated cells were suspended in minimal essential medium (MEM: M 4780; Sigma-Aldrich, St Louis, MO, USA) containing 1.2 mM [Ca²⁺] supplemented with 2.5% fetal bovine serum (FBS; Thermo Fisher Scientific, Cergy-Pontoise, France), 1%

penicillin–streptomycin, 20 mM Hepes (pH 7.6). They were plated on 35 mm, laminin-coated (10 mg ml^{-1}) culture dishes at a density of 10^4 cells per dish and kept at 37°C ($0.5\% \text{ CO}_2$). After 1 h, the medium was replaced by $300 \mu\text{l}$ of FBS-free MEM. To perform Förster resonance energy transfer (FRET) imaging, the medium was replaced by $300 \mu\text{l}$ of FCS-free MEM or transduced with an adenovirus encoding the Epac- SH^{187} FRET-based sensor (Klarenbeek, Goedhart, van Batenburg, Groenewald, & Jalink, 2015) at a multiplicity of infection of 1000 pfu/cell and cells were cultured for 24 h prior to the experiments. All patch-clamp and IonOptix experiments were performed on freshly isolated cardiomyocytes after at least 1 h of FBS deprivation.

2.4 | Electrophysiological experiments

The whole cell configuration of the patch-clamp technique was used to record $I_{\text{Ca,L}}$. Patch electrodes had resistance of $0.5\text{--}1.5 \text{ M}\Omega$ when filled with internal solution containing (in mM): CsCl 118, EGTA 5, MgCl_2 4, Na_2 -phosphocreatine 5, Na_2ATP 3.1, Na_2GTP 0.42, CaCl_2 0.062 (pCa 8.5), Hepes 10, adjusted to pH 7.3. External Cs^+ -Ringer solution contained (in mM): NaCl 107.1, CsCl 20, NaHCO_3 4, NaH_2PO_4 0.8, D-glucose 5, sodium pyruvate 5, Hepes 10, MgCl_2 1.8, CaCl_2 1.8, adjusted to pH 7.4. The cells were depolarized every 8 s from -50 mV to 0 mV during 400 ms. The junction potential was adjusted to give zero current between pipette and bath solution before the cells were attached to obtain a tight gigaseal ($>1 \text{ G}\Omega$). Cell capacitance was obtained by fitting the transient portion of the current response to a 5 mV pulse from -80 mV with a single exponential and calculated from the exponential and the area under the curve obtained. The use of -50 mV as holding potential allowed the inactivation of voltage-dependent sodium currents. Currents were not compensated for capacitance and leak currents. The amplitude of $I_{\text{Ca,L}}$ was measured as the difference between the peak inward current and the current at the end of the depolarization pulses. The current–voltage relationship and steady-state inactivation properties were measured by applying a 200 ms pulse varying from -60 to $+60 \text{ mV}$, followed by a 3 ms repolarization to -50 mV before the 200 ms test pulse to 0 mV . The current density–voltage (I – V) relationships were fitted with a modified Boltzmann equation as follows: $I = G_{\text{max}}(V - V_{\text{rev}})/\{1 + \exp[-(V - V_{0.5,\text{act}})/k]\}$, where I is the current density (in pA pF^{-1}), G_{max} is the maximum conductance (in nS pF^{-1}), V_{rev} is the reversal potential (in mV), $V_{0.5,\text{act}}$ is the midpoint voltage for current activation (in mV), and k is the slope factor (in mV). Steady-state inactivation curves were obtained by normalizing the peak current at each test potential to the maximal current, and fitted with a Boltzmann equation: $I/I_{\text{max}} = (1 - A)/\{1 + \exp[(V - V_{0.5,\text{inact}})/k]\} + A$, where $V_{0.5,\text{inact}}$ is the potential of half-maximal inactivation, k is the slope factor, and A is the amplitude of the non-inactivating $I_{\text{Ca,L}}$ current. The cells were voltage-clamped using patch-clamp amplifiers: either a RK-400 (Bio-Logic, Claix, France) or an Axopatch 200B (Axon Instruments, Inc., Union City, CA, USA) patch clamp amplifier. Currents were analog filtered at 5 kHz and digitally sampled at 10 kHz using a 16-bit analog-to-digital converter (DT321; Data translation, Marlboro, MA, USA or a Digidata 1440A, Axon Instruments) connected to a PC (Dell, Austin, TX, USA).

2.5 | Measurements of Ca^{2+} transients and sarcomere shortening

All experiments were performed at room temperature. Isolated ARVMs were loaded with $1 \mu\text{M}$ Fura-2 AM (Thermo Fischer Scientific) and Pluronic acid (0.012% , Thermo Fischer Scientific) for 15 min in a Ringer solution containing (in mM): KCl 5.4, NaCl 121.6, sodium pyruvate 5, NaHCO_3 4.013, NaH_2PO_4 0.8, CaCl_2 1.8, MgCl_2 1.8, glucose 5, Hepes 10, pH 7.4 with NaOH. Sarcomere shortening and Fura-2 ratio (measured at 512 nm upon excitation at 340 and 380 nm) were simultaneously recorded in Ringer solution, using a double excitation spectrofluorimeter coupled with a video detection system (IonOptix, Milton, MA, USA). Myocytes were electrically stimulated with biphasic field pulses (5 V, 4 ms) at a frequency of 1 Hz as previously described (Mika et al., 2013). Ca^{2+} transient amplitude was measured by dividing the twitch amplitude (difference between the end-diastolic and the peak systolic ratios) by the end-diastolic ratio, thus corresponding to the percentage of variation in the Fura-2 ratio. Similarly, sarcomere shortening was assessed by its percentage variation, which was obtained by dividing the twitch amplitude (difference between the end-diastolic and the peak systolic sarcomere length) by the end-diastolic sarcomere length. Relaxation kinetics were estimated by a non-linear fit of the decaying part of the Ca^{2+} transient and sarcomere shortening traces with the following equation: $X(t) = A\exp(-(t - t_0)/\tau) + A_0$, where t_0 is zero time, A_0 the asymptote of the exponential, A the relative amplitude of the exponential and τ the time constant of relaxation. The maximum first derivative of transients during the deflexion allowed determination of the rising velocities of the signals. All parameters were calculated offline with dedicated software (IonWizard 6x, IonOptix®).

2.6 | FRET imaging

FRET experiments were performed at room temperature 24 h after cell plating. Cells were maintained in a Ringer solution containing (in mM): NaCl 121.6, KCl 5.4, MgCl_2 1.8, CaCl_2 1.8, NaHCO_3 4, NaH_2PO_4 0.8, D-glucose 5, sodium pyruvate 5, Hepes 10, adjusted to pH 7.4 (with NaOH). Images were captured every 5 s using the $\times 40$ oil immersion objective of a Nikon TE 300 inverted microscope connected to a software-controlled (Metafluor, Molecular Devices, Sunnyvale, CA, USA) cooled charge coupled device (CCD) camera (Sensicam PE, PCO, Kelheim, Germany). Cells were excited for 150–300 ms by a xenon lamp (100 W, Nikon, Champigny-sur-Marne, France) using a 440/20BP filter and a 455LP dichroic mirror. Dual emission imaging was performed using an Optosplit II emission splitter (Cairn Research, Faversham, UK) equipped with a 495LP dichroic mirror and BP filters 470/30 (cyan fluorescent protein; CFP) and 535/30 (yellow fluorescent protein; YFP), respectively. Spectral bleed-through into the YFP channel was subtracted using the formula: $\text{YFP}_{\text{corr}} = \text{YFP} - 0.6\text{CFP}$. The concentration–effect curves of Iso on cAMP levels were fitted using the equation $Y = A + (E_{\text{max}} - A)/(1 + 10^{\log\text{EC}_{50} - X})$ where EC_{50} is the apparent

potency, $E_{\max}$ the maximal effect of the agonist and A the minimal percentage CFP/YFP ratio measured.

2.7 | Detubulation of ventricular myocytes

Detubulation was achieved either by inducing osmotic shock with the control solution used for the different experiments (external Cs^+ -Ringer solution prior to patch-clamp or Ringer solution used for Ca^{2+} transient measurement and FRET imaging) containing formamide (1.5 M) for 15 min as described previously (Brette et al., 2002; Kawai et al., 1999) or by preincubating the cells with control solutions containing 300 μM of imipramine for 15 min followed by washout of the CAD.

2.8 | T-tubule imaging

After plating on laminin, myocytes were bathed for 5 min in a Ringer solution and loaded with the lipophilic membrane marker di-4-ANEPPS (2 μM in a Ringer solution for 10 min). After washout, T-tubules were examined by using a confocal microscope (TCS SP8, Leica Microsystems, Wetzlar, Germany) with a $\times 63$ NA 1.20 water immersion objective and the pinhole set to 1 Airy unit. Di-4-ANEPPS was excited at 478 nm and fluorescence collected between 500 and 750 nm. The centre image from each cell was selected for a thresholding analysis using an ImageJ plugin described previously (Heinzel et al., 2002). T-tubule quantification was performed using the ImageJ free software. Images were filtered with a despeckle filter and a median filter, after the background was subtracted with the rolling ball algorithm. The image was then binarized using an auto local threshold, with the Bernsen method (Autothreshold plug-in). The contrast threshold parameter was visually adjusted to ensure the coherence of the binarized image with the original one. A region of interest was defined inside the cell excluding the plasma membrane and then a percentage area was calculated. This value corresponds to the ratio of the number of white pixels over the size of the region multiplied by 100 and thus constitutes an index of the T-tubule density.

2.9 | Statistical analysis

All results are expressed as mean $\pm$ SD. Statistical analysis was performed using Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Normal distribution was tested by a Shapiro–Wilk normality test. For normally distributed data, an unpaired Student's t test was performed. We performed an unpaired t test to determine statistical significance between two groups. Because the data obtained did not follow a normal distribution, a Kruskal–Wallis followed by Dunn's *post hoc* test was used to analyse differences between multiple groups. A χ^2 test followed by Fisher's exact test was used when appropriate. The F -test for model discrimination was used to analyse differences between the concentration–effect curves. Differences with P -values < 0.05 were considered as statistically significant. The number of independent experiments performed is indicated in the figure legends.

3 | RESULTS

3.1 | Imipramine is more efficient and less toxic than formamide in detubulating adult rat ventricular myocytes

Our initial experiments were designed to examine the structure of the T-tubular system in control and adult rat ventricular myocytes treated with either imipramine or formamide, in order to compare the respective ability of these compounds to produce detubulation. To do so, we stained cell membrane with the lipophilic fluorescent dye di-4-ANEPPS. Using confocal microscopy, we visualized the indicator, which highlighted the cell surface on the periphery and T-tubules in the centre of control cells (Figure 1a). We then calculated the percentage of the cell interior occupied by membranes accessible to staining to approximate the T-tubule density. It represented about 20% of the surface of the control cardiomyocytes (Figure 1b, $n = 34$). In contrast, T-tubules were absent in cells treated with imipramine ($n = 32$, $P < 0.001$ versus control) while in formamide-treated cells, these invaginations were indeed decreased ($n = 35$, $P < 0.001$ versus control) but still covered 6% of the total measured area to remain more abundant than in imipramine-treated cells (Figure 1b, $P < 0.01$). Thus, osmotic shock induces partial detubulation while imipramine removes completely the T-tubular system. Furthermore, formamide treatment severely damaged the cell structure, with only ~40% of the cells conserving a rod-shaped appearance while imipramine had no obvious impact on cell morphology (Figure 2a,b, $P < 0.001$ versus formamide). These results suggest that unlike imipramine, the osmotic shock induced by formamide damages the cells with a large proportion dying upon treatment. Similarly to what has been described with formamide (Brette et al., 2002), detubulation induced by imipramine remained 2 h after treatment (data not shown), indicating that it is not reversible (Osman et al., 2016) at least within this time frame. Additionally, when di-4-ANEPPS was incubated prior to imipramine treatment, a striated pattern remained within the cardiomyocyte (data not shown). Thus, with imipramine, T-tubules form vacuoles detached from the sarcolemma within the cell as observed following the osmotic shock evoked by formamide (Brette et al., 2002).

3.2 | Imipramine decreases cell capacitance and $I_{\text{Ca,L}}$ amplitude in adult rat ventricular myocytes but not in atrial cells

Using the patch-clamp technique in the whole cell configuration, we observed a diminution of cell capacitance from 184.2 ± 49.6 ($n = 21$) to 105.2 ± 28.6 pF ($n = 20$; $P < 0.01$) in cells subjected to imipramine (Figure 3a), indicating a loss of nearly 40% of total membrane. A double-pulse protocol allowed the testing of biophysical properties of the L-type Ca^{2+} current recorded in control cells or in cardiomyocytes pretreated with imipramine (Figure 3b,c). $I_{\text{Ca,L}}$ maximal amplitude measured at 0 mV decreased by ~72% from -1556.8 ± 555.1 to -414.1 ± 222.9 pA in detubulated cells confirming enrichment of channels within the T-tubules. Half-activation potential

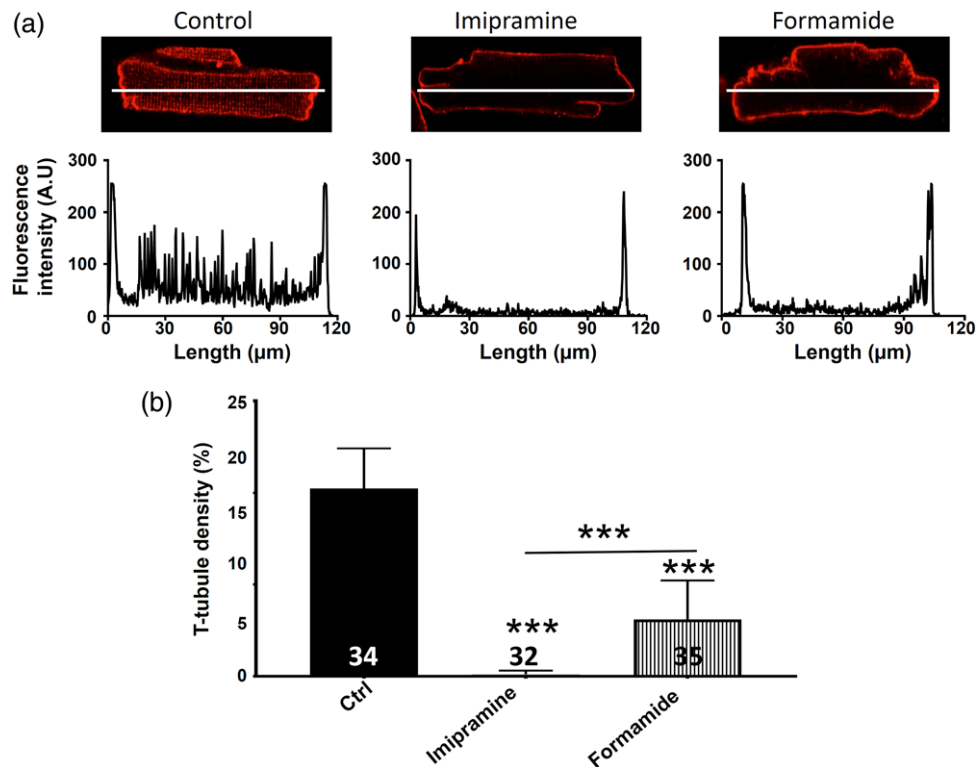


FIGURE 1 Imipramine is more potent than formamide in detubulating adult rat ventricular myocytes. (a) Representative confocal images of adult rat ventricular myocytes subjected or not to a 15 min preincubation with either 300 μ M imipramine or 1.5 M formamide prior to washout and then stained with the lipophilic dye di-4-ANEPPS. The fluorescence intensity profile along the white line in the upper images is shown in arbitrary units in the graphs below. (b) Mean percentage of T-tubules within the cells ($\pm$ SD) evaluated using a technique of ‘thresholding’ and pixel counting. This allowed calculation of the percentage occupied by membrane within a region of interest defined inside the cell (excluding the plasma membrane), which is an index of the T-tubule density. The number of cells obtained from 3 rats is indicated on the graph. Statistical significance using a Kruskal–Wallis test followed by Dunn’s *post hoc* test is indicated, *** $P < 0.001$

was depolarized after imipramine treatment from -13.8 ± 4.1 mV ($n = 21$) in control to -8.9 ± 6.7 mV ($n = 20$, $P < 0.01$) suggesting altered gating of the channels remaining in the sarcolemma. As expected, reversal potential remained unchanged at +59 mV in all tested conditions. In cells treated with imipramine, half steady-state inactivation potential was slightly but significantly shifted towards negative potentials from -24.9 ± 0.9 mV to -26.9 ± 1.3 mV ($n = 20$ –21, $P < 0.01$). Furthermore, in accordance with less Ca^{2+} -dependent inactivation (Argibay, Fischmeister, & Hartzell, 1988), at potentials more positive than +20 mV, $I_{\text{Ca,L}}$ elicited by the test pulse became progressively larger in cells treated with the antidepressant agent (Figure 3c).

Because the majority of adult rat atrial cardiomyocytes lack T-tubule invaginations (Brette et al., 2002; Smyrniak et al., 2010), we tested the effect of imipramine in these cells for potential side effects. Besides, this allowed us to test whether the diminution of $I_{\text{Ca,L}}$ amplitude recorded in imipramine-treated ventricular cardiomyocytes could be the consequence of its known inhibitory effect on cardiac $I_{\text{Ca,L}}$ at micromolar concentration (Isenberg & Tamargo, 1985). Unlike in ARVMs, preincubation of atrial myocytes with imipramine followed by washout caused a small but non-significant effect on membrane capacitance (Figure 4a). Furthermore, imipramine

preincubation had no significant effect on either amplitude or voltage dependence of $I_{\text{Ca,L}}$ in atrial cells (Figure 4b). These results indicate that a 15 min exposure of cardiomyocytes to the cationic amphiphilic drug followed by washout has no direct effect on cell membrane or on LTCCs.

To determine whether the β -AR stimulation of $I_{\text{Ca,L}}$ was affected by imipramine preincubation, ARVMs were subjected to an application of a submaximal concentration of isoproterenol (Iso, 3 nM) and the current recorded using the double-pulse protocol allowing the comparison of the effect of the non-selective β -AR agonist on biophysical properties of the channels in control and detubulated cells (Figure 5a). Maximal conductance (G_{max}) was increased from 0.16 ± 0.04 pS ($n = 21$) up to 0.21 ± 0.04 pS ($n = 15$, $P < 0.001$) in control cells, leading to a 36% increase of $I_{\text{Ca,L}}$ density at 0 mV ($n = 15$, $P < 0.04$) (Figure 5b). Similarly, in detubulated cells, G_{max} was increased from 0.08 ± 0.04 pS ($n = 20$) to 0.13 ± 0.04 pS by Iso ($n = 15$, $P < 0.01$) and $I_{\text{Ca,L}}$ density at 0 mV increased by 22% ($n = 15$, $P < 0.01$) (Figure 5b). The difference observed on $I_{\text{Ca,L}}$ amplitude elicited by the test pulse in cells treated with imipramine was abolished upon Iso treatment and the half inactivation potential remained hyperpolarized at -26.5 ± 1.2 mV in imipramine-treated cells ($n = 15$, $P < 0.01$) compared to -24.5 ± 0.9 mV measured in non-treated cells (Figure 5c).

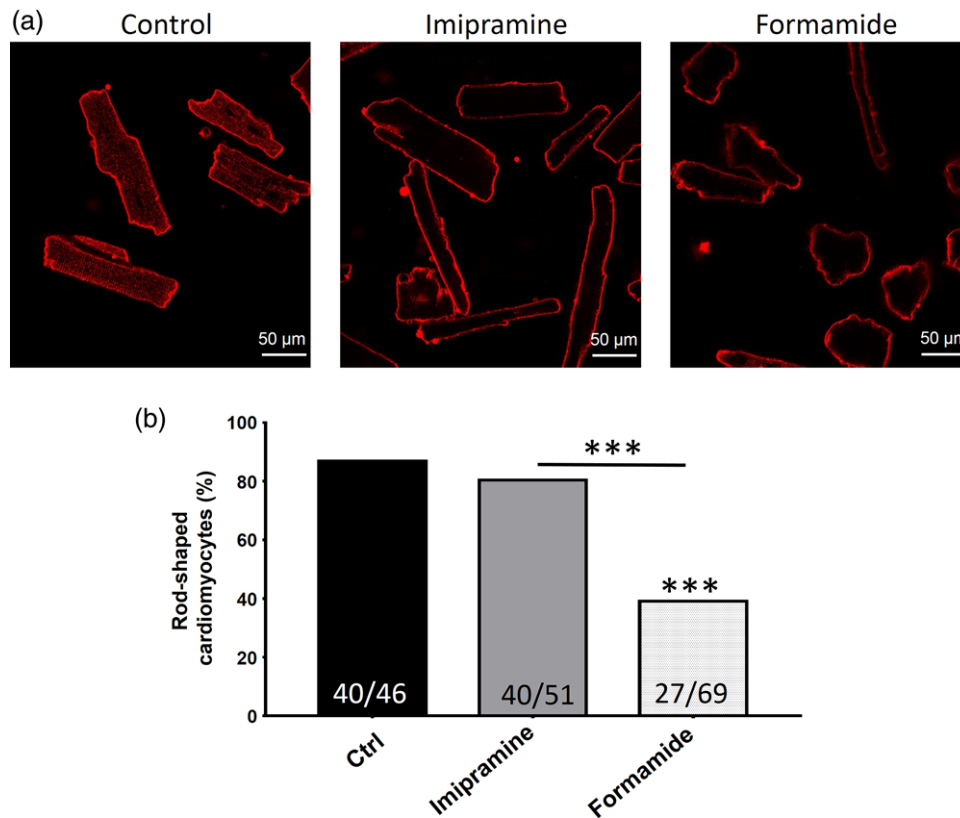


FIGURE 2 Detubulation with imipramine but not with formamide preserves rod shape of adult rat ventricular myocytes. (a) Representative field area confocal images of adult rat ventricular myocytes subjected to a 15 min pre-incubation with a control solution without or with 300 μM imipramine or 1.5 M formamide prior to washout. Cells were then stained with the lipophilic dye di-4-ANEPPS. (b) The mean ($\pm$ SD) percentage of rod-shaped cardiomyocytes. The number of cells obtained from 3 rats is indicated on the graph. Statistical significance using a χ^2 test followed by Fisher's exact test is indicated, *** $P < 0.001$

3.3 | Effect of imipramine treatment on Ca^{2+} transients and contraction of adult rat ventricular myocytes

To test the consequences of detubulation induced by imipramine on ECC, we recorded calcium transients (CaT) and sarcomere shortening (SS) simultaneously in cells loaded with 1 μM Fura-2 and paced at 1 Hz in control conditions and upon β -adrenergic stimulation (Iso, 3 nM) (Figure 6a). Cells pre-incubated with imipramine had an average diastolic sarcomere length of $1.80 \pm 0.04 \mu\text{m}$ ($n = 14$), slightly but significantly increased compared to that of control ARVMs of $1.68 \pm 0.07 \mu\text{m}$ ($n = 14$, $P < 0.001$). The cationic amphiphilic drug drastically decreased mean CaT amplitude from $30.4 \pm 13.9\%$ ($n = 15$) above diastolic Fura-2 ratio in control cells to $8.2 \pm 9.5\%$ ($n = 14$, $P < 0.001$) (Figure 6b). SS was $9.7 \pm 5.3\%$ of diastolic sarcomere length ($n = 15$) in control ARVMs and was also diminished by the imipramine pre-treatment ($2.3 \pm 5.0\%$, $n = 14$, $P < 0.05$) (Figure 6b). In control cells, CaT and SS declined to diastolic levels with average time constants (τ) of 0.18 ± 0.04 and 0.08 ± 0.04 s, respectively ($n = 15$). Decline of CaT was slowed in imipramine-treated cells ($\tau = 0.29 \pm 0.1$ s, $n = 14$) as well as relaxation kinetics ($\tau = 0.43 \pm 0.4$ s, $n = 14$, $P < 0.001$) (Figure 6c). Strikingly, imipramine decreased the rise velocity of CaT from 35.8 ± 23.9 ratio unit s^{-1} ($n = 15$) to 10.8 ± 11.8 ratio unit s^{-1}

($n = 14$, $P < 0.01$) and of SS from $2.4 \pm 1.3 \mu\text{m s}^{-1}$ ($n = 15$) to $3.9 \pm 1.2 \mu\text{m s}^{-1}$ ($n = 14$, $P < 0.01$) (Figure 6d).

In control myocytes, application of a submaximal concentration of Iso (3 nM) slightly increased CaT amplitude and accelerated Ca^{2+} return to diastolic levels ($\tau = 0.1 \pm 0.04$ ratio unit s^{-1} , $n = 15$, $P < 0.001$; Figure 6b,c). Upon Iso, a tendency for acceleration of the rising phase of CaT was observed (57.6 ± 39.9 ratio unit s^{-1} , $n = 15$; Figure 5d). The β -AR stimulation produced inotropic effects, increasing SS to $15.9 \pm 4.7\%$ ($n = 15$, $P < 0.05$) with slight but not significant lusitropic effects (Figure 6b,c). In cells pre-treated with imipramine, Iso increased CaT amplitude ~ 2.3 -fold ($n = 14$, $P < 0.05$), and it remained significantly decreased compared to CaT recorded in control cells exposed to Iso (Figure 6b, $n = 14$, $P < 0.05$). Noticeably, in imipramine-treated cells, while Iso increased the rise velocity of SS ($n = 15$, $P < 0.01$) similarly to what was observed in Ctrl cells ($n = 14$, $P < 0.05$), it failed to speed up the onset of CaT. However, as expected, the decay of CaT became ~ 3 times faster ($n = 14$, $P < 0.0001$) and SS relaxation increased 6-fold ($n = 14$, $P < 0.01$) to become similar to those of control cells upon β -AR stimulation (Figure 6c). Another striking effect of imipramine pre-treatment was to favour Ca^{2+} perturbances (Figure 6e). While we did not observe spontaneous Ca^{2+} activities in control cells, either under basal conditions or upon Iso, more than 40% of the detubulated cells with the antidepressant agent exhibited

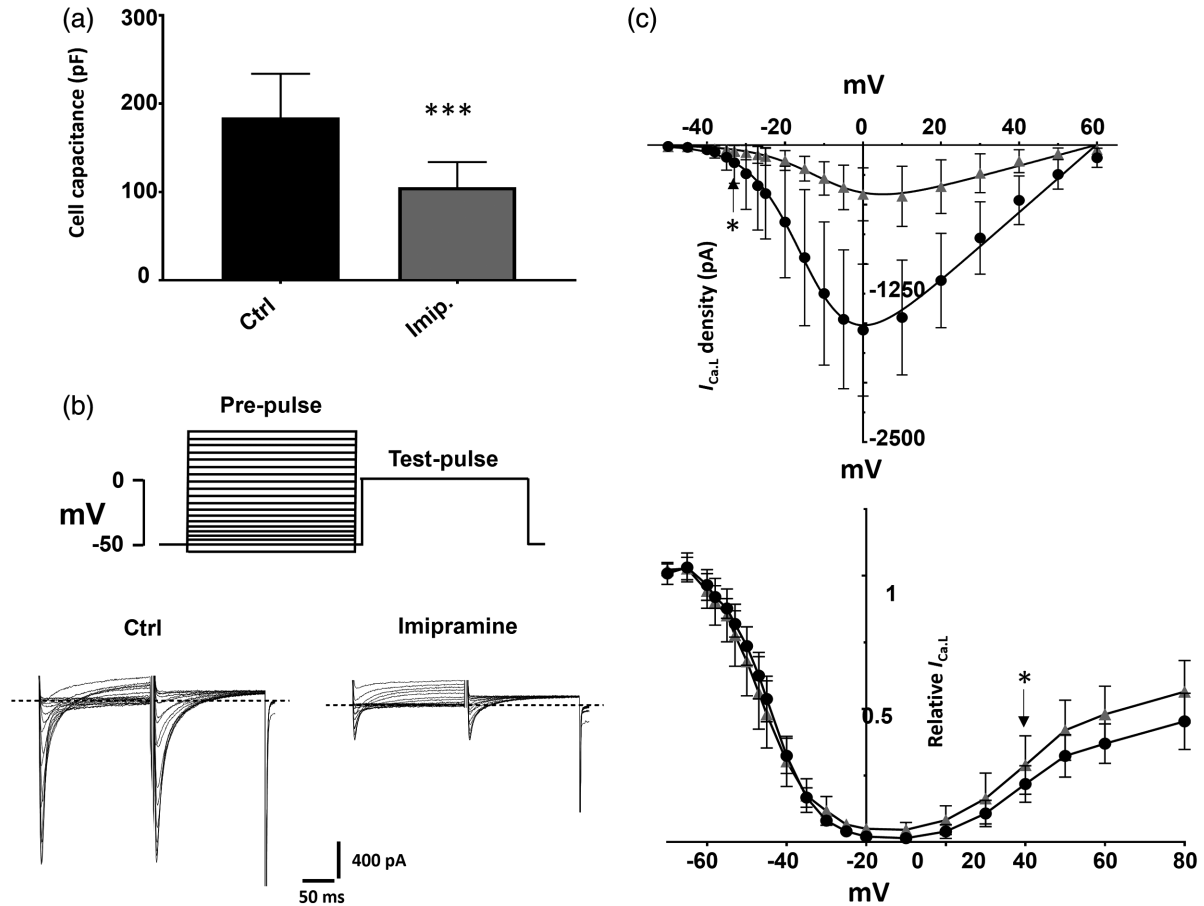


FIGURE 3 Effect of imipramine on cell capacitance and $I_{Ca,L}$ measured in adult rat ventricular myocytes. (a) The black bar represents the mean value ($\pm$ SD) of cell capacitance obtained from 21 control cardiomyocytes, the grey bar the mean capacitance of 20 cells pretreated with 300 μ M imipramine for 15 min before washout. (b) L-type Ca^{2+} currents were elicited using the double-pulse protocol from a holding potential of -50 mV (inset). Superimposed recordings of $I_{Ca,L}$ obtained during the pre-pulses in control or pretreated with imipramine myocytes are shown. (c) Mean variation ($\pm$ SD) current-voltage (left panels) and steady-state inactivation (right panels) relationships for $I_{Ca,L}$ in control (circles, $n = 21$) and imipramine (triangles, $n = 20$) cardiomyocytes. These results were obtained in cells isolated from 5 animals. Statistical significance using an unpaired t test is indicated, * $P < 0.05$, *** $P < 0.001$ (points that occur after the time point indicated on the curves by the arrows are significant)

pro-arrhythmic events ($n = 15$, $P < 0.01$), a proportion reaching nearly 80% upon β -AR stimulation ($n = 14$, $P < 0.0001$ versus control cells in Iso).

3.4 | Effect of Imipramine treatment on intracellular cAMP levels produced upon a β -adrenergic stimulation in adult rat ventricular myocytes

Next, we compared cAMP levels in control ventricular cardiomyocytes and in cells subjected to imipramine pretreatment by overexpressing the FRET-based sensor Epac-S^{H187} (Klarenbeek et al., 2015) in ARVMs using a recombinant adenovirus. As shown in Figure 7a, in a control cell, a brief application of Iso (15 s, 100 nM) increased transiently the CFP/YFP ratio. The mean variation of FRET ratio produced by Iso in control cells was $78.9 \pm 20.4\%$ ($n = 11$) and this was reduced by 27.8% in cells subjected to imipramine ($n = 11$, $P < 0.01$) (Figure 7b), without any significant alteration in the time course of the signals. We next subjected cells to sustained increasing concentrations of Iso from 1 nM up to 300 nM (Figure 7c). The

FRET ratio increased in both conditions, but the plateau value was decreased in detubulated cells ($n = 17$ –28, $P < 0.001$). While apparent potency (EC_{50}) remained identical (EC_{50} was 19.6 ± 19.9 nM in control versus 18.3 ± 22.6 nM in imipramine), the maximal effect of Iso was reduced from $105.7 \pm 37.8\%$ to $76.9 \pm 22.5\%$ by imipramine. Thus, from these results we can conclude that detubulation with a high concentration of the antidepressant agent imipramine diminishes by ~27% the cAMP production, suggesting that the majority of cAMP production upon a non-selective β -AR stimulation occurs outside the T-tubules.

4 | DISCUSSION

4.1 | Detubulation of ventricular myocytes with imipramine

Our study confirms that imipramine disrupts T-tubules from the surface membrane, and thus can be used as a new detubulating agent.

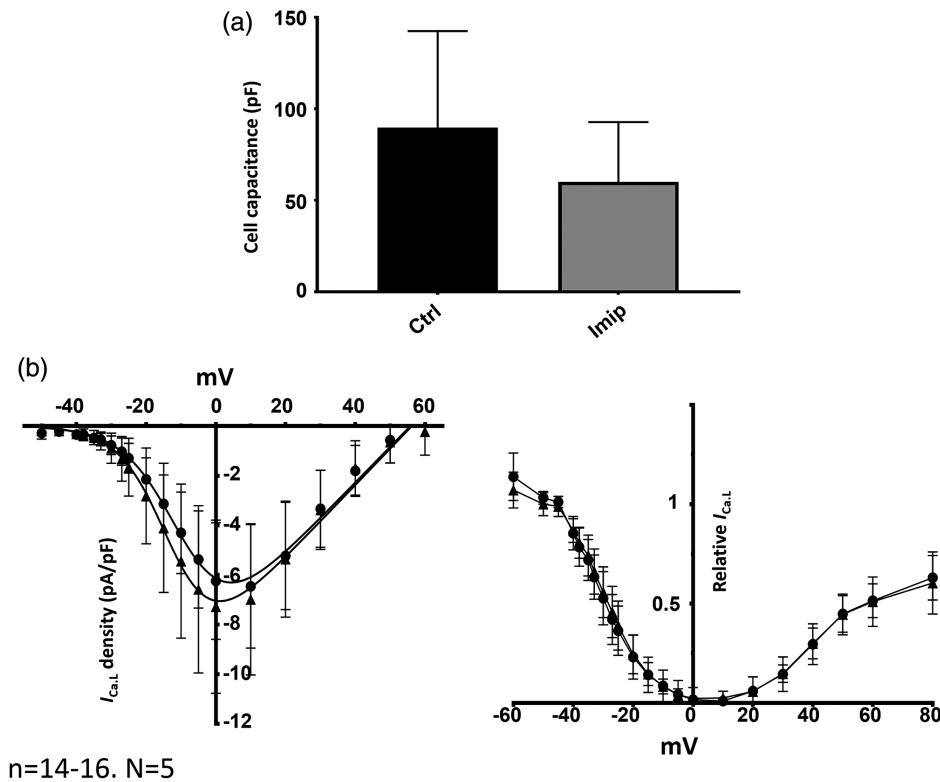


FIGURE 4 Imipramine affects neither cell capacitance nor $I_{Ca,L}$ in rat atrial cardiomyocytes. (a) Mean ($\pm$ SD) of cell capacitance was measured in control atrial cardiomyocytes (black bar, $n = 14$) or atrial cells pre-treated with imipramine 300 μ M for 15 min prior to washout (grey bar, $n = 16$). (b) Mean ($\pm$ SD) current–voltage (left panels) and steady-state inactivation (right panels) relationships of $I_{Ca,L}$ elicited using a double-pulse protocol from a holding potential of -50 mV in control (circles, $n = 14$) and imipramine-treated (triangles, $n = 16$) cardiomyocytes obtained from 5 rats

This new method presents several advantages compared to formamide treatment. First, the cells conserve their rod shape after imipramine pretreatment, suggesting that unlike the detubulation evoked by an osmotic shock, imipramine-induced detubulation preserves cell viability. Additionally, this new detubulation method is more efficient since we did not observe tubular system staining with di-4-ANEPPS after imipramine treatment while we observed that the interior of the surviving cells after formamide treatment still exhibited a few tubules as shown previously (Kawai et al., 1999). More efficient detubulation is further suggested by the $\sim 40\%$ decreased cell capacitance, which is higher than the 26–30% diminution produced by formamide in ARVMs that was reported previously (Brette & Orchard, 2006; Brette, Salle, & Orchard, 2006; Bryant et al., 2014; Kawai et al., 1999). Furthermore, this diminution better approximates the T-tubular membrane fraction ($\sim 49\%$) predicted by computer modelling (Pasek et al., 2008). Besides, since the majority of adult rat atrial myocytes lack T-tubules (Brette et al., 2002; Smyrniak et al., 2010) the absence of a significant effect on cell capacitance allows the exclusion of any direct effect of imipramine on cell membrane other than detubulation. Another advantage of this new method is that, unlike formamide, imipramine does not detubulate cardiomyocytes by producing an acute osmotic shock, which induces cell volume changes (Kawai et al., 1999) known to evoke electrical remodelling (Ravens, 2003) and signalling pathway activation (Gonano et al., 2014). Imipramine is a tricyclic antidepressant which contains a hydrophobic ring structure and a hydrophilic side chain with a charged

cationic amine group, and hence it is part of the class termed CADs. CADs are one of the largest classes of pharmaceutical compounds and these drugs are known to accumulate at the internal leaflet of cell membranes (Sheetz & Singer, 1974), where they bind to the negative charges of PI(4,5) P_2 via their cationic amine group (Halliwell, 1997). Therefore it constitutes an alternative to the drastic osmotic shock produced by formamide to detubulate cells, interfering with the binding of the phospholipid to BAR domain-containing proteins (Suetsugu, Kurisu, & Takenawa, 2014). However, it is worth noticing that it may interfere with many cellular functions since PI(4,5) P_2 not only is crucial for membrane tubulation (Hofhuis et al., 2017; Inaba et al., 2016), but is an actor in endocytosis and exocytosis (Suetsugu et al., 2014), regulates ion channels (Royal, Tinker, & Harmer, 2017), and is involved in Ca^{2+} signalling and intracellular pathways (Berridge, 2009). In ventricular myocytes, bridging integrator 1 (BIN1) is likely to interact with PI(4,5) P_2 because it contains a BAR domain and thus may allow the correct folding of the T-tubules and formation of the dyad (Fu et al., 2016; Hong et al., 2014). However, it is probably not the sole phospholipid binding protein playing this role in heart since dysferlin, which recruits PI(4,5) P_2 to shape the T-tubule system in skeletal muscle (Hofhuis et al., 2017), is also expressed in the heart (Han et al., 2007) where it may also participate in such architectural organization. The role of PI(4,5) P_2 in the scaffolding of T-tubules of cardiac cells is further supported by our results which demonstrate that a CAD disconnects these invaginations from surface cell membranes.

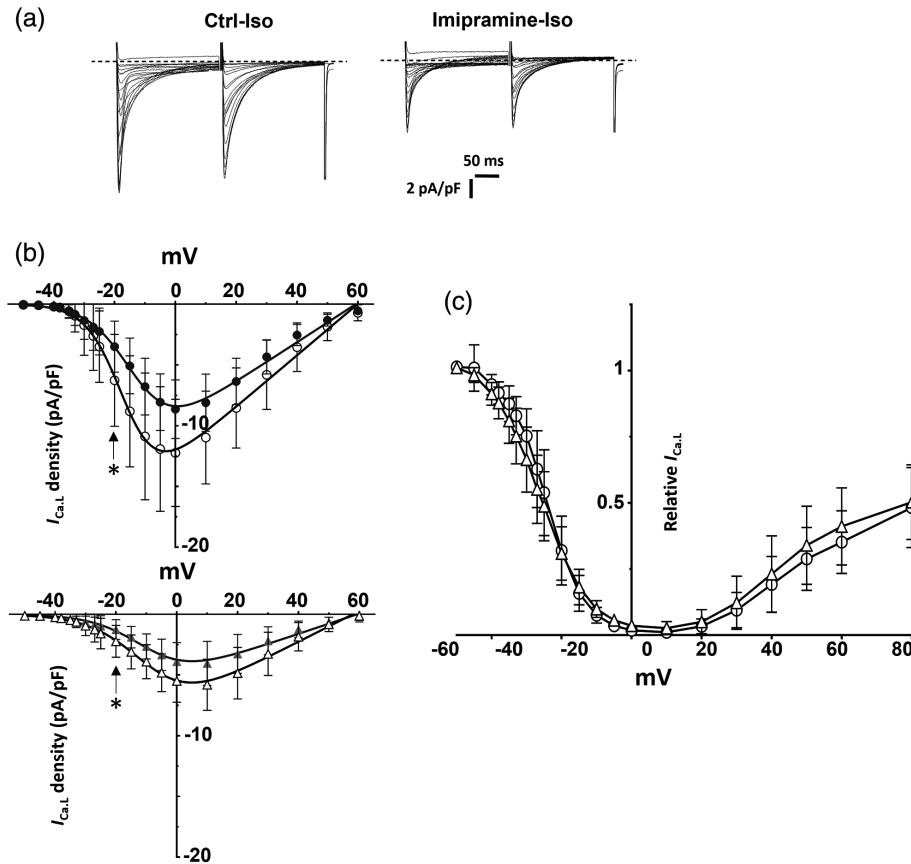


FIGURE 5 Imipramine does not affect the β -adrenergic stimulation of $I_{Ca,L}$ in rat ventricular cardiomyocytes. (a) Superimposed recordings of $I_{Ca,L}$ obtained during the pre-pulses in control or pretreated with imipramine myocytes upon β -AR stimulation by isoproterenol (Iso, 3 nM). (b,c) Mean ($\pm$ SD) current-voltage (b) and steady-state inactivation (c) relationships of $I_{Ca,L}$ elicited using a double-pulse protocol from a holding potential of -50 mV recorded under basal conditions (filled symbols, $n = 20$ – 21) or upon Iso (open symbols $n = 14$ – 17) in control (circles) and imipramine-treated (triangles) cardiomyocytes obtained from 5 rats. Statistical significance using an unpaired t test is indicated, $*P < 0.05$ (points that occur after the time point indicated on the curves by the arrows are significant)

Interestingly, phospholipids are altered in pressure overload hypertrophied hearts (Reibel et al., 1986), in myocardial infarction (Nasa et al., 1997), in the cardiac tissue from mice treated with doxorubicin (Moulin et al., 2015) or catecholamines (Emilsson & Gudbjarnason, 1981). Thus, decreased $PI(4,5)P_2$ within membranes may constitute a link with the decreased density of these invaginations in heart failure (Hong & Shaw, 2017), a hypothesis which requires further work but is supported by our present results.

4.2 | Effects of imipramine pretreatment on ECC in ventricular myocytes

While detubulation of cells with imipramine reduced by $\sim 40\%$ cell capacitance, we observed a $\sim 72\%$ decrease of $I_{Ca,L}$ amplitude similar to the diminution by 60–76% of $I_{Ca,L}$ previously recorded after formamide treatment (Brette et al., 2006; Bryant et al., 2014; Kawai et al., 1999). This is in line with an enrichment of the T-tubules with LTCCs (Kawai et al., 1999) and suggests that T-tubules concentrate ~ 4 times more channels than the rest of the membrane; this is less than previously estimated, probably due to previous underestimation of cell capacitance using formamide (Kawai et al., 1999; Pasek et al., 2008).

Imipramine is well known to inhibit $I_{Ca,L}$ in cardiac cells and this effect was demonstrated to be reversible even at micromolar concentrations (Isenberg & Tamargo, 1985). The lack of effect on $I_{Ca,L}$ amplitude or its voltage dependence when measured in atrial cells indicates that the direct effect of imipramine on the channel was fully reversed after washout of the drug during our detubulating procedure. This also confirms that the decreased Ca^{2+} entry measured in ventricular cells is due to an effective loss of channels concentrated within the invaginations. Interestingly, $I_{Ca,L}$ activation was significantly shifted toward depolarized voltages after detubulation. This is compatible with channels being more tightly regulated by protein kinase A in T-tubules (Brette, Rodriguez, Komukai, Colyer, & Orchard, 2004a) where caveolin-3 facilitates basal activity of the kinase (Bryant et al., 2014; Timofeyev et al., 2013) and the localization of LTCCs (Bryant et al., 2018). Besides, voltage dependence of steady-state inactivation was shifted towards negative potentials, suggesting differential regulation of surface membrane and T-tubule $Ca_v1.2$ channels. This may occur via specific association with different variants of auxiliary subunits such as β or $\alpha_2\text{-}\delta$ which modulates inactivation of LTCCs (Shaw & Colecraft, 2013). Furthermore, at positive potentials, the degree of $I_{Ca,L}$ inactivation which correlates with the Ca^{2+} -dependent

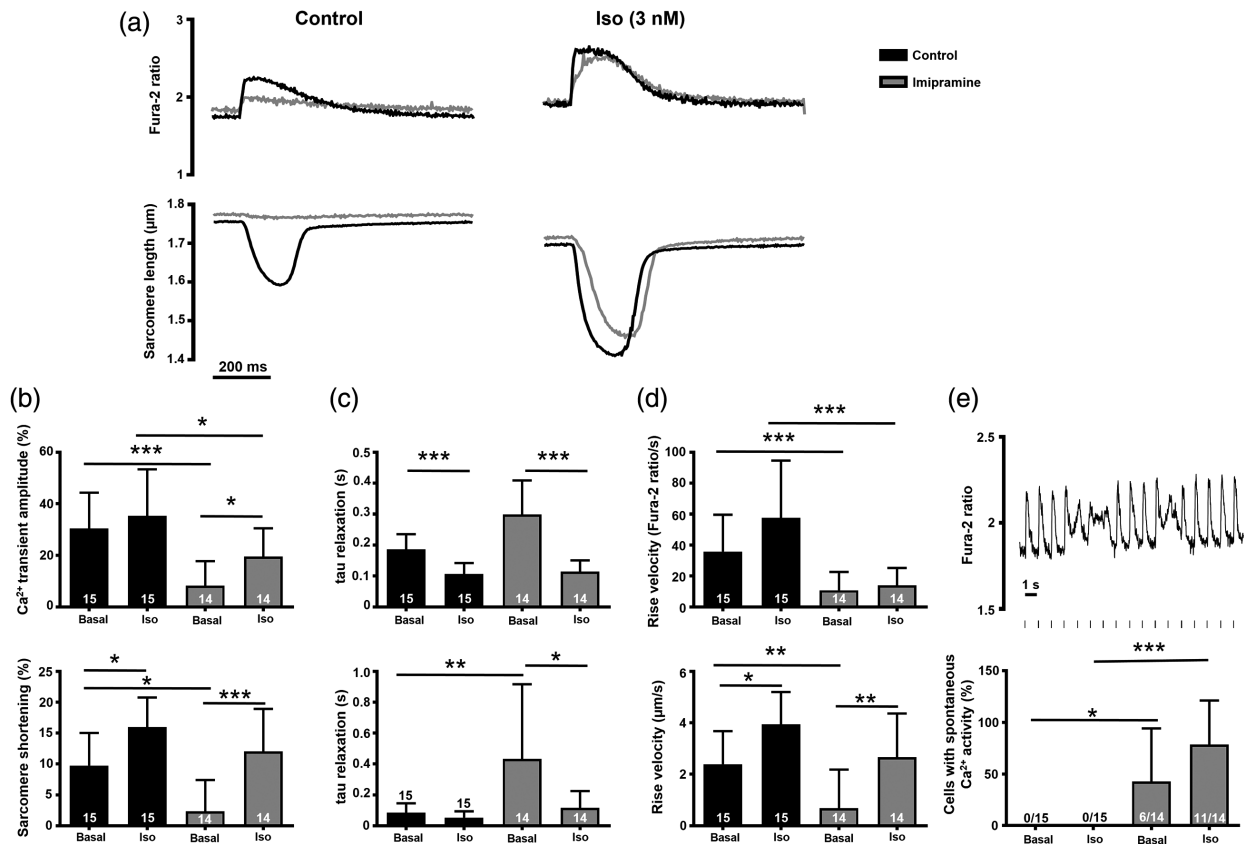


FIGURE 6 Effect of the detubulation induced by imipramine on ECC in adult rat ventricular myocytes. (a) Representative traces of Ca²⁺ transients and sarcomere shortening in Fura-2 loaded control or imipramine-detubulated ventricular cells paced at 1 Hz. This protocol was repeated under basal conditions (Ctrl) and upon β -AR stimulation by isoproterenol (Iso, 3 nM). (b) Mean amplitude ($\pm$ SD) of Ca²⁺ transients (upper graph) and sarcomere shortening (lower graph) in Ctrl (black bars) or imipramine-treated cells (grey bars). (c,d) Average relaxation kinetics ($\pm$ SD) (c) and mean ($\pm$ SD) rise velocity (d) of Ca²⁺ transients (upper graphs) and sarcomere shortening (lower graphs). (e) Proportion of cells exhibiting pro-arrhythmic spontaneous and asynchronous Ca²⁺ releases. Number of cells is indicated inside each bar representing the mean of 5 independent experiments. Statistical significance is indicated, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. A Kruskal–Wallis test followed by Dunn's *post hoc* test was used to analyse Ca²⁺ transients and sarcomere amplitude. A χ^2 test followed by Fisher's exact test was used to compare the percentage of arrhythmic cells

inactivation (Argibay et al., 1988) was less pronounced in detubulated cells. This is in accordance with less negative feedback of Ca²⁺ released from the SR on channels in the sarcolemma (Brette, Salle, & Orchard, 2004b) and the decreased transient amplitude we observed in myocytes pretreated with imipramine. Imipramine pretreatment diminished by two-thirds CaT amplitude and thus decreased SS. This is equivalent to the previously reported ~70% decreased CaT amplitude induced by formamide detubulation (Brette et al., 2002, 2004b; Kawai et al., 1999) and is probably due to the decreased Ca²⁺ entry in the cardiomyocyte and decreased SR Ca²⁺ load in detubulated cells (Brette, Despa, Bers, & Orchard, 2005). Furthermore, the rise velocity of CaT was impaired and detubulated cells exhibited spontaneous CaT and Ca²⁺ alternans confirming that the T-tubules are critical to ensure normal and rapid, synchronized Ca²⁺ release (Brette et al., 2002, 2005; Caldwell et al., 2014; Frisk et al., 2016; Oyehaug et al., 2013). Moreover, the tendency to decreased rate decline of CaT in imipramine-treated cells is compatible with decreased Na⁺–Ca²⁺ exchange proteins known to localize in the T-tubules (Despa, Brette, Orchard, & Bers, 2003; Kieval, Bloch, Lindenmayer, Ambesi, & Lederer,

1992) and decreased SERCA2 activity pumping Ca²⁺ back to the SR due to diminished Ca²⁺ concentration gradient. Altogether, our results demonstrate that the cells pretreated with imipramine exhibit the characteristic features of detubulated cells.

4.3 | Effect of imipramine on β -adrenergic cAMP signaling and regulation of the ECC

FRET imaging revealed an ~27% decrease of intracellular cAMP levels elicited by brief or sustained non-selective β -AR stimulation in imipramine-treated cells. This diminution is in line with decreased cAMP production sites by detubulation, due to the loss of β -AR and AC present in the tubular system (Nikolaev et al., 2010; Timofeyev et al., 2013). The rather low impact of detubulation on β -AR-generated cAMP might result from the fact that upon Iso application, cAMP mainly emanates from sarcolemmal β_1 -ARs, which produce highly diffusible signals throughout the cell (Nikolaev et al., 2010), in contrast to the β_2 -ARs, which reside within the invaginations (Nikolaev et al., 2010; Timofeyev et al., 2013). Furthermore, the recovery of cAMP

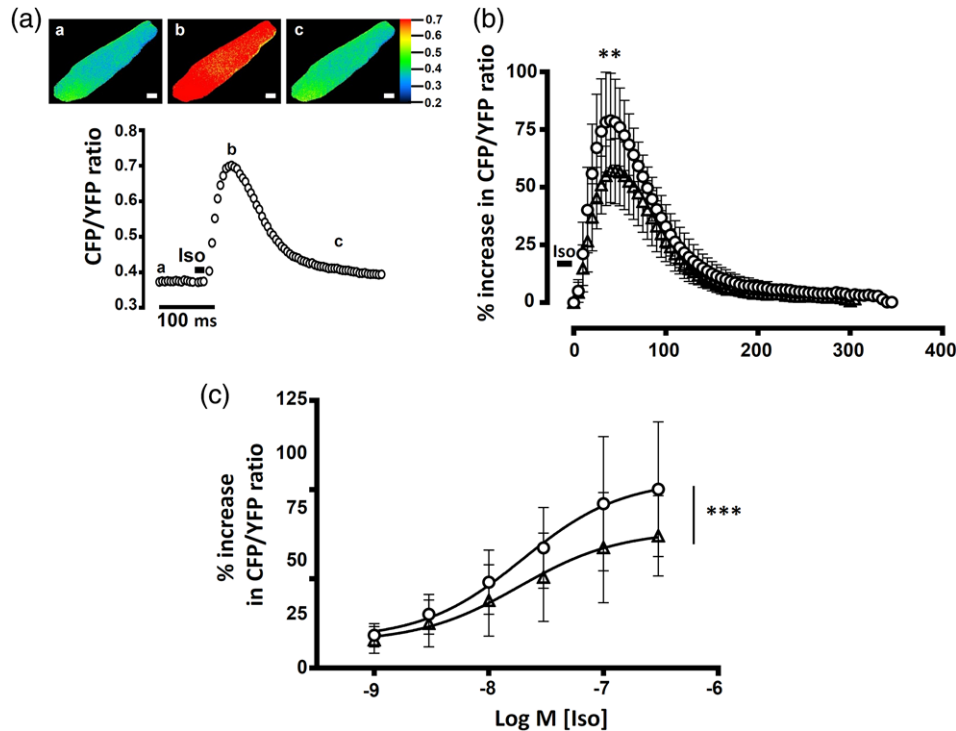


FIGURE 7 Effect of imipramine on intracellular cAMP levels upon β -adrenergic stimulation. (a) Representative time course of cAMP levels in a cardiomyocyte expressing the Epac-S^{H187} FRET-based sensor upon a brief β -adrenergic stimulation with isoprenaline (Iso, 15 s, 30 nM). CFP/YFP ratio was evaluated by the normalized cyan fluorescent protein (CFP) over yellow fluorescent protein (YFP) ratio emitted upon CFP excitation at 440 ± 20 nm. Pseudo-colour images of the CFP/YFP ratio were recorded at the times indicated by the letters on the graphs. Scale bar is 10 μ m. (b) Mean ($\pm$ SD) variation with time of the CFP/YFP ratio upon a pulse of Iso measured in control (circles, $n = 14$) and imipramine-treated (triangles, $n = 16$) cardiomyocytes. (c) Concentration–response curves for the effect of isoprenaline on the CFP/YFP ratio measured in control cells (circles) and imipramine-treated cardiomyocytes (triangles) ($n = 17$ –28). An unpaired t test was used to test the difference in maximal FRET ratio variation and an F -test to compare the concentration–response curves in both conditions. Data represents the mean $\pm$ SD of the number of cells obtained from 5 rats. Statistical significance is indicated, ** $P < 0.01$, *** $P < 0.001$

signals, which depends on phosphodiesterase activity (Leroy et al., 2008), was not impacted by detubulation. This is probably due to the fact that the enzymes remained attached to the vacuoles disconnected from the sarcolemma after imipramine treatment (data not shown). Consequently, the non-selective β -AR stimulation in detubulated cells still increases $I_{Ca,L}$ because the majority of the LTCCs at the surface membrane are still coupled to β_1 -AR/AC (Timofeyev et al., 2013). As a result, Iso was able to restore CaT amplitude, and thus contraction, by increasing not only Ca^{2+} entry but probably also SR Ca^{2+} load by promoting the protein kinase A phosphorylation of phospholamban and consequently SERCA2 activity, which remains in detubulated cells with formamide (Brette et al., 2004a). The noticeable lusitropic effects we observed upon Iso application suggests that this is also the case in imipramine-treated cells. However, the rise velocity of CaT was not augmented by Iso in detubulated cells, contrary to what was reported in myocytes treated with formamide (Brette et al., 2004a). This discrepancy might be due to the incomplete detubulation following the osmotic shock while imipramine removes totally the tubular system. Furthermore, imipramine cells also exhibited Ca^{2+} homeostasis perturbations upon Iso, further underlying the crucial role of T-tubules in obtaining rapid and synchronized CICR, even upon β -AR stimulation.

5 | CONCLUSIONS

Although the concentration of imipramine we used here to detubulate cardiomyocytes is much higher than the plasmatic levels reached with therapeutic doses ($\sim 1 \mu$ M), our results suggest that a disorganization of T-tubules may contribute to the clinical cardiotoxic effects of the antidepressant drug, especially upon overdose (Marshall & Forker, 1982). Indeed it can evoke heart failure in dog (Lucas et al., 1992) and intoxication with this tricyclic antidepressant (TCA) has been associated to cardiac arrhythmias, depression of myocardial contractility, hypotension and cardiac arrest (Marshall & Forker, 1982), which might have been caused, at least partly, by the detubulation of the cardiomyocytes. Furthermore, besides providing new potential insights into the cardiotoxicity of high doses of imipramine, our results validate a new alternative detubulation technique of cardiomyocytes using an agent which is known to interfere with PI(4,5)P₂. This method is more efficient than the osmotic shock induced by formamide and importantly has no impact on cell viability but allows recapitulation of the hallmarks of detubulation on ECC. Thus, we propose imipramine as a novel utensil in the toolbox of cardiac physiologists whose aim is to study the role of T-tubules in cardiac cells.

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COMPETING INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

J.L. conceptualized and designed the work. J.L., G.V. and R.F. participated in the drafting of the article. A.B., M.B., I.B., P.L., H.B.M. and J.L. participated in the acquisition, analysis and interpretation of data for the work. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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